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# A first-in-class, non-invasive, immunodynamic biomarker approach for precision immuno-oncology

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**ABSTRACT**

Non-invasive, immuno-dynamic, biomarkers positioned in cancer patient's blood milieu with immuno-oncological applications are rare. We recently established a "first-in-class" serum functional immuno-dynamics status (sFIS) assay, wherein *in vitro* assessment of serum-induced myeloid NFkB and/or interferon (IFN) response-signaling can be performed to "mimic" *in situ* patient's serum immune-biology. This modality has clear implications for anticipating patient prognosis and immunotherapy-relevant stratification.

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Cancer immunotherapy has revolutionized the field of oncology, however not all cancer-types or patient subgroups respond well, thereby limiting its application. Beyond multi-modal combinatorial immunotherapy, high precision biomarkers that can differentiate responders from non-responding patients can hugely improve the outlook of immunotherapy.[1] Multiple biomarkers have been investigated to address this need, e.g., high tumor mutational burden, high immune checkpoint expression and/or high T cell infiltration.[2] Unfortunately, the methods for detecting these parameters are mostly focusing on the tumor micro-environment, thereby making such biomarkers invasive and hard to measure at multiple timepoints.[3] To overcome these deficiencies, current research is focusing on the development of high-precision, noninvasive biomarkers, detectable in patient's serum, plasma, or blood. Recently, several sera/plasma-associated prognostic or predictive biomarkers have been assessed in cancer patients. These include specific serum-associated cytokines/metabolites andperipheral immune cell-subsets. However, such features are either not independent prognostic biomarkers or are less reliable for rapid screening purposes,[4] because the biomarker approach for these is almost always more quantitation-driven, with qualitative assessment being entirely dependent on bioinformatics or computational approaches. However, since bioinformatic or computational approaches cannot reliably model the non-linear, dynamic, immune signaling, this creates a major bottleneck for biomarker validation and interpretation across heterogeneous patient subgroups. These deficiencies have created a critical unmet need for immunodynamic biomarkers that can be noninvasively assessed and can capture the heterogeneity of highly variable tumor and/or patient immune-status.

In a recent study from our lab,[5] we have conceptualized an innovative assay to overcome this bottleneck. Through a multitude of advanced computational immunology, bioinformatics, biomedical literature mining-algorithms, and systems biology approaches, we established that the top two broadest inflammatory pathways, which can capture the entirety of cancer patient's serum-immunology, were: Nuclear Factor kappa-light-chain-enhancer of activated B (NFkB)-signaling and the interferon (IFN)-stimulated response element (ISRE)/IFN-stimulated genes' (ISG) signaling.

Of note, although the immune-biology of these two pathways is well known, their role as biomarkers based on few discrete (downstream or upstream) cytokines or chemokines, has been rather challenging with often contradictory clinical outcomes. For instance, it has been shown that breast cancer patients with a lower expression of the IFN signature genes have a better overall survival (OS) (wherein, autophagy seems to modulate IFN-signaling[6,7]), whereas lung cancer patients responding to radiotherapy showed elevated Interferon Beta 1 (IFNB1) cytokine presence in serum, post-radiotherapy treatment, which might correlate to a better OS.[8] We believe these contradictions may arise from heterogeneity of the IFN/ISG response in clinical settings, which may not be sufficiently captured via a few cytokines/chemokines or limited genetic signatures and are subject to autoregulation in inflammatory contexts. Furthermore, the NFkB-

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signaling pathway also displays a huge complexity. NFKB signaling follows an oscillatory pattern, making it cumbersome to pinpoint the net effect via proxy cytokines/chemokines.[9] This is not surprising since NFkB can be expressed in all cell types and has a lot of upstream activators as well as downstream targets that intensely cross-talk with almost all cellular signaling pathways. Consequently, depending on the context, proxy biomarkers for NFkB/IFN-response can be either positive or negative (or even null) prognostic, as they are only capturing a small snapshot of the entire pathway.

To this end, we used publicly available "The Cancer Genome Atlas" (TCGA) cohorts of various major human cancers to test the prognostic/mechanistic impact of tumoral NFkB/IFN-signaling genetic signatures. Multiple cancer types showed a correlation of the NFKB or the IFN signatures with bad or good prognosis, respectively. This was cumulatively captured by selected cancer-types, especially the TCGA ovarian cancer (OV) cohort, wherein we established that tumor-level NFkB-signaling genetic-signature is negative prognostic, while IFN response genetic-signature is positive prognostic. Nevertheless, these results were still only on the tumoral level and their link with the periphery needed further investigation for putative immunodynamic biomarker validation. This gap-in-knowledge was a major motivation for our biomarker discovery approach.

To this end, for the first time, we were able to combine both IFN and NFKB responses in an immunodynamic biomarker analysis to overcome the unreliability of individual cytokines. This first-in-class assay was put in an ideal framework for examining these two pathways (Figure 1). We utilized myeloid cells as "signaling hub" since they were shown to have the highest enrichment of both the IFN/ISG and the NFkB-signaling. To conceptualize the serum functional immunodynamic status (sFIS) assay, we used the THP1 myeloid cell line containing two reporter constructs that can be induced via (1) the interferon Induced Protein with tetratricopeptide repeats 2 (IFIT2) promoter (upstream of secreted LUCIA luciferase gene) and (2) the NFKB consensus transcriptional response element (upstream of secreted embryonic alkaline phosphatase (SEAP)). Herein, these myeloid reporter cells were supposed to sense the cytokines/factors in sera and respond via the appropriate pathway. Using this approach, we aimed to establish an "active" link between tumor-level and peripheral immune-status across unrelated cohorts of OV – a reverse translational approach seldom demonstrated for most noninvasive immuno-oncology biomarkers.

Stunningly, the previously established tumoral trends for IFN and NFkB response were successfully confirmed on the level of patient periphery (serum) in two independent OV patient's cohorts, one consisting of 98 archived serum samples derived from 32 randomly selected OV-patients and the TRANS-IOTA trial comparing the 699 diagnostic (baseline) serum samples from multicenter European patients with benign (404 patients) or borderline (90 patients) ovarian lesions vs. ovarian malignancy (205 patients) patients. Remarkably, OV-patients with shorter OS or progression-free survival (PFS) had significantly higher serum-induced (si) NFkB-signaling in our sFIS assay (driven by pro-metastatic, wound healing-like signaling), whereas OV-patients with longer OS or longer PFS had serum that induced higher IFN responses. Surprisingly, this IFN response was driven by

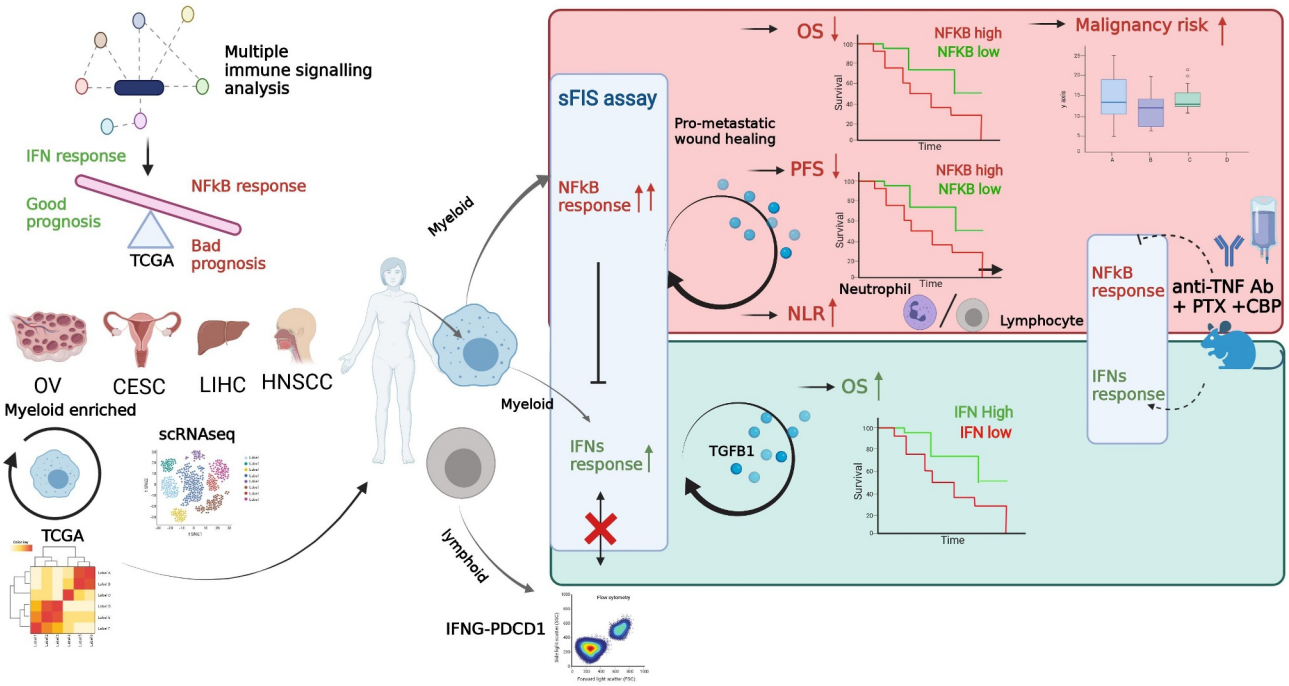


**Figure 1.** Schematic overview of the development of the sFIS assay. Through multiple immune signaling analysis, the IFN and NFKB pathway were pinpointed as good and bad prognostic in multiple cancer types at the tumor-level, with their enrichment being most evident in the myeloid compartment. By incorporating these signaling pathways in a myeloid reporter assay we were able to determine, in a large OV patient's cohort, that high NFkB response predicts lower OS and PFS and a higher NLR and malignancy risk. Contrastingly, a high IFN/ISG response correlated with a higher OS. Surprisingly, the IFN/ISG response did not synergize with lymphoid IFNG or PDCD1-CD274 serum-enrichment. Additionally, the sFIS assay was able to predict response of anti-TNF therapy in combination with chemotherapeutics. Ab; antibody, CBP; carboplatin CESC; Cervical squamous cell carcinoma, HNSCC; Head and neck squamous cell carcinoma, LIHC; Liver hepatocellular carcinoma, NLR; neutrophil-to-lymphocyte ratio, OS; overall survival, OV; Ovarian cancer, PFS; progression free survival, PTX; paclitaxel, scRNAseq; small conditional RNA sequencing, sFIS; serum functional immunodynamic status, TCGA; The Cancer Genome Atlas.

supra-high concentrations of Transforming Growth Factor Beta 1 (TGFB1) and did not align with immunosuppressive IFNG-PDCD1-CD274 signaling driven concomitantly by Interferon Gamma (IFNG), Programmed Cell Death 1 (PDCD1) and CD274 (also called Programmed Cell Death 1 Ligand 1 or PD-L1). Mucin 16 (MUC16, also known as Cancer Antigen 125 or CA125), the standard-of-care tumor burden biomarker for OV, was relatively inconclusive albeit trending toward shortened OS. Interestingly, si-NFkB response positively correlated to the neutrophil-to-lymphocyte ratio (NLR), a well-established negative prognostic biomarker. Furthermore, we also analyzed the capability of our sFIS assay to distinguish malignancy risks. Indeed, combining serum CA125 and si-NFkB signaling was highly capable at differentiating ovarian malignancy from benign/borderline ovarian-lesions. These results together established that si-NFkB signalling$^{HIGH}$si-IFN/ISG response$^{LOW}$ is a predominant status of most ovarian cancer patients' periphery and this status has a negative prognostic immuno-stratification with pro-malignant tendencies. Interestingly, we recently showed that the utility of our sFIS assay is also extendable to other cancer-types. For example, our assay showed that non-small cell lung carcinoma (NSCLC) patients treated with stereotactic body radiation therapy (SBRT) had elevated plasma-induced IFN/ISG response activity after treatment, associating with significantly better PFS.[10]

Finally, a major hurdle in biomarker research is the lack of reproducible 'reverse translational' association between human and preclinical animal models' immune status. This creates severe bottlenecks in using clinically validated biomarkers to guide selection of immunotherapeutic combinations in animal models, to eventually anticipate a clinical roadmap for biomarker-driven applications of novel immunotherapeutic combinations. To overcome this, we conceived a murine version of the sFIS assay using murine myeloid cell (J774 cells). In this manner, we were able to predict the ability of chemo-immunotherapy (paclitaxel-carboplatin combined with antibody against Tumor Necrosis Factor or TNF) to create a pro-immunogenic peripheral environment based on high si-IFN/ISG response and low si-NFkB response in an orthotopic murine metastatic OV model.[11] This proved that the sFIS assay can be used for biomarker-driven designing of complex immunotherapeutic regimens.

In conclusion, we believe that our sFIS assay can tremendously help in better understanding the peripheral immune responses of cancer patients, and their clinico-immunological and prognostic features. By giving an overview of potential regulation or dysregulation in the peripheral immune compartment, we can facilitate monitoring, immuno-stratification, and immunotherapeutic decision-making. Hence, this study establishes the first proof of concept for biomarker assays using peripheral immunodynamics in cancer patients.

## Disclosure statement

The sFIS assay is currently the subject of an ongoing PCT patent application.

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